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Effects of exposure and previous exposure to copper on growth of veliger larvae and survivorship of *Mytilus edulis* juveniles

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ABSTRACT: Exposure of *Mytilus edulis* L. (Mollusca: Bivalvia) to 8 ppb (ppb = µg l^{-1}) added copper during the veliger larval or post-larval (spat) stages has no significant effects on survivorship or shell growth. However, previous exposure to 8 ppb added copper during the embryo stage significantly increases veliger growth rate and decreases spat survivorship in a population from the Menai Strait, Wales, UK, in which this level of copper causes a significant increase in embryo abnormality. No such effects of pre-exposure are seen in a population from the Westerschelde, The Netherlands, where 8 ppb added copper does not constitute a stress on the embryo. These 'pre-incubation' (embryo exposure) effects far outweigh any direct influence of 8 ppb copper exposure during the later stages and have a substantial effect on estimates of the toxicity of copper over the life cycle of *M. edulis*.

KEY WORDS: Mussels Larvae Copper · Pollution · Pre-exposure · Long-term · *Mytilus edulis* · The Netherlands · United Kingdom

INTRODUCTION

Davenport & Redpath (1984) reviewed the literature on the effects of copper on the mussel *Mytilus edulis* L. Copper is an essential element for *Mytilus* spp. but it is among the most toxic of metals when in excess (Viarengo 1989). An increase in copper concentration of 15 ppb results in mortality of 50% of adult *M. edulis* in about 30 d (Manley 1980). Relatively unpolluted coastal waters have copper levels within the range 2 to 5 ppb (Davenport & Redpath 1984; ppb = µg l^{-1}), while concentrations of over about 30 ppb are confined to the immediate locality of pollution sources (Lewis & Cave 1982). Intracellular protective substances such as metallothioneins (Viarengo et al. 1980, 1981a, b, 1988, 1989, Roesijadi et al. 1982, Harrison et al. 1988) act by removing copper from solution by forming relatively stable, insoluble complexes with it.

Exposure of mussels to low levels of copper can increase their resistance to higher copper concentrations (Davenport & Manley 1978, Roesijadi & Fellingham 1987), probably by stimulating the synthesis of metallothionein (Viarengo et al. 1980, Viarengo 1989).

Embryo development appears to be the most copper-sensitive stage in the life cycle of *Mytilus edulis*; the EC_{50} ('50% Effective Concentration', the concentration which causes a 50% reduction in the specified parameter within the specified period) for production of normal 'D' larvae has been estimated to be 5.8 ppb added copper by Martin et al. (1981) and 7.9 ppb added copper by Barnes (1989).

The veliger larva has the highest resistance to copper of any stage in the life cycle of *Mytilus edulis*, exhibiting a 15 d LC_{50} ('50% Lethal Concentration', the concentration which causes 50% mortality within the specified period) of 500 ppb Cu and a growth rate EC_{50} of about 200 ppb Cu (Beaumont et al. 1987). The reduction in copper tolerance to the adult level is gradual and completed when a shell length of about 5 mm is reached (Hoare & Davenport 1994). Sub-lethal effects occur at much lower concentrations; Beaumont

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et al. (1987) found a small but significant reduction in veliger shell growth at 20 ppb added copper.

Ringwood (1992) found evidence for physiological adaptation of veligers of the bivalve *Isognomon californicum* to cadmium exposure and suggested that this was due to metallothionein induction. Inheritance and induction of copper metallothioneins in the embryos and veligers of *Mytilus edulis* have yet to be investigated. Roesijadi et al. (1982) exposed mature *M. edulis* to mercury for 2 wk just prior to spawning and found mercury metallothioneins in the resulting 'D' larvae, but, since a small amount of mercury was found to have been passed on to the larvae, there is some uncertainty as to whether this metallothionein was inherited or autogenous.

One aim of the experiments described in this paper was to provide information on possible differences in physiological response to copper challenge among populations of *Mytilus edulis* which have differing pollution exposure histories. In order that the results might be relevant to the situation in the field, long-term experiments were carried out using a level of copper within the range reported for coastal waters (Lewis & Cave 1982).

MATERIALS AND METHODS

Cultures. The treatment regimes (Table 1) began just after fertilisation; the embryo is the most pollution-sensitive stage in the life cycle of *Mytilus edulis* (Martin et al. 1981) and therefore would probably be under the greatest selection pressure. The treatment regimes were designed to allow the effects of copper treatment at each stage on that and subsequent stages to be evaluated. There were 2 replicate cultures (a and b) per treatment group.

The source populations were chosen from sites where *Mytilus edulis* occurs intertidally in the absence of the closely related hybridising species *M. galloprovincialis* and *M. trossulus* Gould. (Gosling 1992). The 'clean' site source population was from Gallows Point (53° 15' N, 4° 06' W), Menai Strait, Wales, UK, a relatively uncontaminated open coast site without significant riverine influence. The 'polluted' site source population was from Veerhaven (51° 26' N, 4° 03' E), Westerschelde, The Netherlands. The Westerschelde, part of the Rhine-Meuse delta system, forms the estuary of the River Schelde. Mussels from Veerhaven and other sites in the Westerschelde have been shown to have high tissue concentrations of mercury, lead, chromium, arsenic, nickel, cadmium, copper, zinc (Luten et al. 1986, Stronkhorst 1992), polychlorinated biphenyls (PCB) and pesticides (Hummel et al. 1990, Stronkhorst 1992).

Table 1. Copper exposure at 3 stages of development of 6 treatment groups of laboratory-reared *Mytilus edulis*. +: copper exposure; –: control conditions

Developmental stage (duration)	Treatment group 1	2	3	4	5	6
Embryo (3 d)	–	–	–	+	+	+
Veliger (3–4 mo)	–	–	+	–	+	+
Post-settlement (4–5 mo)	–	+	+	–	–	+

All experiments were carried out in accordance with the provisions of the Sea Fisheries (Shellfish) Act 1967 as varied by the Molluscan Shellfish (Control of Deposit) (Variation) Order 1983, S.I. no. 159.

Menai Strait and Westerschelde veliger cultures spawned on 16 July 1991 and 13 July 1990, respectively, were grown on to settlement using the methods of Bayne (1965); see Hoare et al. (1995; this issue) for details of the spawning techniques and embryo cultures .

For each veliger culture, up to 20 000 normal 3 d old larvae ('D' larvae) were transferred to a sterilised glass 5 l beaker to give about 5 'D' larvae ml^{-1}, with varying proportions of abnormal 3 d old larvae. *Pavlova lutheri* (Droop) Green and *Rhinomonas reticulata* (Lucas) Novarino were added to give 40 and 10 cells μl^{-1} respectively.

Copper (as $CuCl_2 \cdot 2H_2O$) solution was diluted with 0.2 µm filtered UV-sterilised seawater (FSW) before being added to the appropriate treatment groups to give a concentration of 8 ppb added copper in these groups; all other treatment groups were exposed to background copper levels (approximately 3 ppb throughout the course of the experiment). The larvae were grown on using standard larval rearing methods (Beaumont et al. 1988). At the start of the experiments potentiometric stripping analysis (Jagner 1982, Redpath 1985) was used to verify that the actual copper concentrations achieved were similar to the desired levels. The results of this analysis indicated that the copper-treated cultures were, on average, exposed to 7.3 ppb (standard deviation 5.4 ppb) Cu more than the control cultures, in agreement with Beaumont et al. (1987) who demonstrated that under standard larval rearing conditions copper levels do not fall by more than 25% during the intervals between changes. Significant early mortality is a common feature of *Mytilus edulis* veliger cultures, and the larvae were therefore counted again when they were 2 wk old and the culture volumes adjusted to maintain 5 normal larvae ml^{-1}.

The shell lengths of the mussels in all of the cultures derived from one of the source sites were assessed using the photomicrographic method of Beaumont &

Budd (1982) when the first pediveligers appeared in the fastest growing culture derived from that site.

The cultures were reared through and beyond metamorphosis as follows. Each culture was transferred to a sterilised, high-sided, 170 µm mesh, plastic sieve which fitted closely into the 5 l glass beaker This allowed settled and unsettled larvae to be kept in the same culture (Beaumont et al. 1988). FSW, copper and algal rations were renewed 3 times per week as previously, except that algae were supplied at double the previous concentrations. Those larvae not retained by the 170 µm sieve were retained during changing in a 45 µm sieve as before.

When fewer than 1% of the larvae still passed through the 170 µm sieves (at an age of about 8 wk) these sieves were transferred from the 5 l glass beakers to sterilised 20 l plastic crates, such that each crate held 3 sieves at the appropriate copper concentration. This transfer to crates facilitated water changing, which continued to be carried out 3 times per week. *Pavlova lutheri* and *Rhinomonas reticulata* continued to be supplied at 80 and 20 cells μl^{-1} respectively.

When it appeared that all mussels within each sieve had settled and reached a shell length of at least 500 µm (at an age of about 3½ mo), the cultures were subjected to the final set of conditions (Table 1). They were grown on in these conditions until they reached a mean size of about 3 mm, a total culture duration of about 8 mo. The effect of treatment on survivorship to this time was estimated but counts were only made where there was a visible difference in survivorship between treatments.

Data analysis. Where possible, analysis of variance (Sokal & Rohlf 1981) was used to test the raw or transformed data for statistical significance. Where data did not fit the assumptions for parametric analysis the non-parametric Kruskal-Wallis and Mann-Whitney tests (Siegel 1956) were used.

RESULTS

Menai Strait

Although there was significant variation in survivorship between replicates within treatments, there were no significant effects of treatment on mortality between 3 and 13 d old. Mean overall survivorship was 35%. No increase in the number or size of empty shells, i.e. in mortality, was observed during the remainder of the veliger stage.

The shell length measurements (Table 2) indicated that there was a significant ($p < 0.05$) difference between treatments in size at 27 d old, above and beyond the difference ($p < 0.001$) between replicates. This difference in growth rates was caused by after effects of different treatments during the embryo stage rather than by different treatments during the veliger stage itself. There was a significant ($p < 0.05$) difference between those groups which had identical treatments at the veliger stage but different copper treatments at the embryo stage. The mean shell length at 27 d old was 203 µm for those which were exposed to 8 ppb added copper as embryos but only 168 µm for those which underwent embryonic development under control conditions. Cultures which had been exposed to 8 ppb added copper during the veliger stage were on average slightly, but not significantly, smaller than those which were cultured at background copper levels during the veliger stage but which had experienced identical embryo treatments.

Table 2. *Mytilus edulis*. Mean shell lengths of Menai Strait veliger larvae cultured under different copper exposure regimes. The standard deviations are given in parentheses. a and b are replicate cultures. Measurements were taken when the cultures were 27 d old from photomicrographs and were of between 13 and 94 larvae per culture. For further explanation of treatment regimes see Table 1

Treatment	Culture	Shell length (µm)
No copper	1a	171 (39)
	1b	148 (30)
	2a	166 (29)
	2b	188 (34)
	Treatment mean:	170
8 ppb Cu at veliger stage	3a	170 (28)
	3b	161 (37)
	Treatment mean:	165
8 ppb Cu at embryo stage	4a	237 (40)
	4b	178 (31)
	Treatment mean:	214
8 ppb Cu at both embryo and veliger stages	5a	187 (38)
	5b	180 (41)
	6a	215 (33)
	6b	198 (49)
	Treatment mean:	200

As mentioned above, there were no signs of differential mortality between treatments during the veliger phase. However, when these cultures had reached the spat stage there were obvious differences in spat densities between the treatments, and so at the end of the experiment the juveniles were counted (Table 3). The total survivorships are shown in Table 3. Analysis indicates that neither veliger nor post-settlement treatment had a significant effect on survivorship. However, copper exposure at the embryo stage caused significantly ($p < 0.002$) increased mortality between the veliger stage and the end of the experiment.

Table 3. *Mytilus edulis*. Survivorships to the end of the experiment in Menai Strait mussel cultures raised under different copper treatment regimes, given both as absolute numbers and as percentages of numbers surviving in the cultures at earlier stages. For further explanation of treatment regimes see Table 1

Treatment	Culture	Survivorship			
		Number	As %13 d old veligers	As % 'D' larvae	As % eggs
No copper	1a	447	4.5	2.2	1.3
	1b	1133	13.5	5.7	2.7
	Treatment mean:	790	9.0	4.0	2.0
8 ppb Cu post-settlement	2a	700	12.7	3.5	1.7
	2b	182	3.6	0.9	0.4
	Treatment mean:	441	8.2	2.2	1.1
8 ppb Cu post-settlement and as veligers	3a	509	5.7	2.5	1.2
	3b	921	20.5	4.6	2.5
	Treatment mean:	715	13.1	3.6	1.9
8 ppb Cu at embryo stage	4a	153	2.4	0.9	0.3
	4b	62	1.1	0.3	0.1
	Treatment mean:	107.5	1.8	0.6	0.2
8 ppb Cu as embryos and veligers	5a	138	1.8	0.7	0.3
	5b	37	1.2	0.2	0.1
	Treatment mean:	87.5	1.5	0.5	0.2
8 ppb Cu at all stages	6a	48	0.6	0.2	0.1
	6b	149	2.3	1.0	0.3
	Treatment mean:	99	1.5	0.6	0.2

Copper stress at the embryo stage appears to have far reaching effects. Mussel cultures which experience 8 ppb added copper as embryos grow faster during the veliger stage and suffer greatly increased mortality thereafter, probably during metamorphosis and settlement. These pre-incubation effects far outweigh any direct influence of copper exposure during these later stages.

Westerschelde

There were no significant effects of treatment on survivorship to 14 d old, although there were significant differences ($p < 0.01$) among replicates within treatments. There were no signs of any additional mortality before settlement.

The length measurements (Table 4) taken at 19 d old indicate that there were no significant differences in size just before settlement.

As with the Menai Strait veliger cultures, those treatment groups which had experienced 8 ppb added copper during the veliger stage or control conditions during the embryo stage were always smaller than those which had experienced the alternative conditions at these stages, but which otherwise received identical treatment. However, these differences were not statistically significant. Survivorship to the end of the experiment was high in all cultures, with no differences apparent among the treatments, although no counts were made.

DISCUSSION

No conclusions should be drawn from the large differences in mean growth rates of the control groups between the Menai Strait- and Westerschelde-derived laboratory populations because the experiments were carried out at different times and thus had differing water and food qualities in addition to the innate differences between the populations. It could be argued that the Menai Strait- and Westerschelde-derived laboratory populations were subjected to differing lengths of exposure to copper during the veliger stage because of their differing growth rates. However, they were exposed for the same *developmental* period. In order that both the duration and the developmental timing of exposure be constant, it is necessary to have identical growth rates in differing cultures; this is a constant problem in studies of this type. In this case it was considered more reasonable to gear experimental exposures to developmental events than to imposed durations.

The effects of copper on the embryo stage of these cultures are detailed in Hoare et al. (1995).

The experiments do not display any significant direct effect of veliger exposure to 8 ppb added copper on

Table 4. *Mytilus edulis*. Mean shell lengths of Westerschelde veliger larvae cultured under different copper exposure regimes. The standard deviations are given in parentheses. Measurements were taken when the cultures were 19 d old from photomicrographs and were of between 36 and 119 larvae per culture. For further explanation of treatment regimes see Table 1

Treatment	Culture	Shell length (µm)
No copper	1a	225 (15)
	1b	214 (23)
	2a	187 (23)
	2b	198 (22)
	Treatment mean:	206
8 ppb Cu at veliger stage	3a	192 (27)
	3b	195 (22)
	Treatment mean:	193
8 ppb Cu at embryo stage	4a	226 (22)
	4b	220 (23)
	Treatment mean:	223
8 ppb Cu at both embryo and veliger stages	5a	209 (19)
	5b	210 (23)
	6a	205 (21)
	6b	199 (22)
	Treatment mean:	206

veliger growth or on survivorship after the 'D'-larva stage.

There is a significant difference between the Menai Strait and Westerschelde cultures in the effects of embryonic copper treatment on later growth and survivorship. It appears that where copper has exhibited a significant stress on the embryos of a culture, i.e. in the 8 ppb copper-treated Menai Strait cultures, the surviving population of morphologically normal 'D' larvae grow significantly faster ($p < 0.05$) up to metamorphosis but then experience significantly higher mortality ($p < 0.002$). Such effects are not seen when embryos are exposed to 8 ppb added copper where this does not appear to constitute a stress, i.e. in the Westerschelde cultures.

A possible hypothesis which might explain the Menai Strait results is that copper exposure at the embryo stage may have sub-lethal, or rather deferred-lethal, effects, in addition to the short-term induction of lethal abnormalities. These effects might be in the form of damage to presumptive adult tissues, or might involve a modification of veliger physiology such that larvae lay down a lesser reserve of lipid, which would allow faster growth while leaving too little stored energy for the successful completion of metamorphosis. Ringwood (1992) showed that cadmium caused significant reductions in veliger mass growth of the Hawaiian bivalve *Isognomon californicum* at concentrations far lower than those required to produce a significant reduction in shell growth. Metamorphosis in *Mytilus edulis* appears to occur only when organic matter, i.e. tissue dry mass, attains a weight of over 45% of the total dry mass of the larva (Lucas et al. 1986). It is therefore possible that small increases in metal concentrations may affect metamorphosis. Some idea of the effects of copper at the different stages can be gained from the survivorships to the juvenile stage under different treatments. The magnitude of the lipid fraction of the pre-settlement dry weight would give an indication of the reserves accumulated to fuel metamorphosis (Holland & Spencer 1973).

The effects of copper stress at the embryo stage on the later stages of the Menai Strait cultures meant that, although 8 ppb added copper produced a mean abnormality/mortality of only 49% within the 3 d exposure period (Hoare et al. 1995), mean survivorship to the juvenile stage in these cultures was only 12% of that found in cultures which had experienced control conditions as embryos. Putative pollution selection in the Westerschelde for resistance to copper toxicity at the embryo stage may therefore also have important repercussions at later life stages.

The effect of copper treatment of Menai Strait *Mytilus edulis* embryos on later life stages appears to be an amalgam of reduced survivorship and hormesis (Stebbing 1982); 8 ppb added copper causes faster cleavage (Hoare unpubl.), reduced embryonic normality, faster veliger growth and greatly reduced survivorship through metamorphosis to the juvenile stage, independently of later treatment. Such a fundamental effect of early treatment may indicate that copper affects the cytoplasmic maternal developmental determinants, which direct molluscan ontogeny. Pre-incubation effects, though considerable in this case, are little studied. Bayne (1972) found that nutritional stress on adult *M. edulis* led to increased rates of developmental abnormality in the offspring, perhaps because of reduced inheritance of energy (Bayne et al. 1978). Bayne et al. (1975) found that stress on parents reduced veliger growth rates, while Helm et al. (1973) found that the offspring of food-stressed oysters (*Ostrea edulis*) had reduced survivorship through metamorphosis and settlement, as well as reduced rates of veliger growth. These studies agree with the copper study described here in that factors which affect early development have long-reaching effects, but disagree in that they did not reveal any hormesis.

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